(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 July 2002 (04.07.2002)

PCT

(10) International Publication Number
WO 02/051351 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/BE01/00220

(22) International Filing Date:
21 December 2001 (21.12.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0031448.4 22 December 2000 (22.12.2000) GB

(71) Applicant *(for all designated States except US)*: **K.U.LEUVEN RESEARCH AND DEVELOPMENT** [BE/BE]; Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DECKMYN, Hans** [BE/BE]; Lemingstraat 1, B-3210 Linden (BE). **CAUWENBERGHS, Nancy** [BE/BE]; Heerbaan 130, B-1840 Londerzeel (BE). **VANHOORELBEKE, Karen** [BE/BE]; Kortrijkstraat 84, B-8550 Zwevegem (BE).

(74) Common Representative: **K.U.LEUVEN RESEARCH AND DEVELOPMENT**; Dr. Roelants Ivo - IPR Officer, Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/051351 A2

(54) Title: ANTITHROMBOTIC VON WILLEBRAND FACTOR (VWF) COLLAGEN BRIDGING BLOCKERS

(57) Abstract: The present invention clearly demonstrates that vWF-collagen interaction plays an important role in acute platelet-dependent arterial thrombus formation and that blockade of vWF-collagen interaction can induce complete abolition of thrombus formation in the injured and stenosed baboon femoral arteries. Accordingly, a blocker of vWF-collagen can be used as a compound for the prevention of acute arterial thrombotic syndromes or to manufacture medicines to prevention of acute arterial thrombotic syndromes.

Antithrombotic von Willebrand factor (vWF) collagen bridging blockers

Karen Vanhoorelbeke, Nancy Cauwenberghs,

and Hans Deckmyn

BACKGROUND OF THE INVENTION

Damage of an arterial vessel wall leads to platelet adhesion, aggregation and ultimately may result in thrombosis. These events are known to contribute to the development of occlusive syndromes in the coronary, cerebral and peripheral vascular system, as well as restenosis and intimal hyperplasia that occur after angioplasty, atherectomy and arterial stenting (1;2). In both thrombosis and reocclusion, platelets adhere to the subendothelium of damaged blood vessels through an interaction with von Willebrand factor (vWF) that forms a bridge between collagen, a component of the damaged vessel wall and the platelet glycoprotein Ib (GPIb) (3). This reversible adhesion or tethering of the platelets at high shear rate is followed by a firm adhesion through the collagen receptors (GPIa-IIa; GPIV,...)(4) resulting in platelet activation and release of ADP, thromboxane, and serotonin. These in turn activate additional platelets and trigger the conformational activation of the platelet GPIIb/IIIa receptor, leading to fibrinogen binding and finally to platelet aggregation (5). Ultimately, a platelet-initiated thrombus is formed.

The search for anti-platelet drugs in the prevention of thrombosis has recently focused on the blockade of the GPIIb-IIIa receptor and on the inhibition of the vWF-GPIb axis. The best characerized drugs are antibodies and peptides that block the binding of adhesive

proteins to GPIIb-IIIa which have been tested in animal models and of which many are being tested in clinical trials and/or are used in the clinic (6-8). Also compounds that interfere with the vWF-GPIb axis inhibit thrombus formation in various animal models. The GPIb/IX/V complex consists of 4 different polypeptides GPIbα, GPIbβ, GPIX and GPV which are all members of the leucine-rich protein family (9;10). The N-terminal domain of the GPIbα polypeptide contains the vWF binding site (11). vWF is composed of several homologous domains each covering different functions : it interacts through its A1 domain mainly with the GPIb/V/IX complex (12), whereas its A3 domain predominantly interacts with fibrillar collagen fibers (13;14). Compounds that interact with GPIbα, like the GPIb-binding snake venom proteins echicetin and crotalin (15;16), an anti-guinea pig GPIb antibody (17;18), a recombinant A1 domain fragment (VCL) (2;23) and recently an anti- human GPIb antibody (19) or compounds that bind to vWF like anti-A1-vWF-monoclonal antibodies (mAbs) (20;21), aurin tricarboxylic acid (ATA) (22) are inhibiting *in vivo* thrombus formation.

Specific blockade of the vWF-collagen interaction in vivo has not yet been demonstrated but could be a novel strategy for the prevention of thrombus formation in stenosed arteries. We here describe for the first time the antithrombotic effect of a murine anti-human vWF mAb82D6A3, known to bind to the A3-domain and to inhibit vWF binding to fibrillar collagens type I, III and calf's skin collagen but not to collagen VI (24), Vanhoorelbeke et al., 2000b).

The present study aimed to evaluate the antithrombotic efficacy of mAb 82D6A3 in baboons by using a modified Folts' model, where cyclic flow reductions (CFRs) due to thrombus formation and its dislodgment are measured in an artery following intimal damage and placement of a critical stenosis to reduce the lumen diameter (25).

ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

EXAMPLES

Materials.

Human placental collagen type I and III and calfskin type I were purchased from Sigma (St. Louis, MO). The collagens were solubilized in 50 mmol/L acetic acid and subsequently dialyzed against phosphate-buffered saline PBS (48 hours, 4°C) to obtain fibrillar collagen. The phage display library with the random hexapeptides flanked by cysteine residues was obtained from Corvas (Gent, Belgium), the pentadecamer phage display peptide library was a kind gift of Dr. G. Smith (University of Missouri, Colombia, Mo). vWF was purchased from the Red Cross (Belgium). The SpI proteolytic fragment and recombinant A3-domain were kind gifts of Drs. JP Girma (INSERM 134, Paris) and Ph. G. de Groot (Utrecht, The Netherlands).

Purification of mAb 82D6A3

mAb 82D6A3 was obtained from a cell line, that has been deposited with the Belgian Collections of Micro-organisms, under accession number LMBP 5606CB and was purified from ascites by protein A chromatography.

Preparation of 82D6A3 F(ab) fragment.

82D6A3-F(ab) was prepared by digestion with papain. Briefly, 5 mg Ab was digested with 50 µg papain (Sigma) in the presence of 10 mmol/L cysteine and 50 mmol/L EDTA (37°C, overnight). The F(ab) was purified by protein A affinity chromatography (Pharmacia, Roosendaal, The Netherlands) and purity was checked by SDS-PAGE.

Surgical Preparation

Seven baboons of either sex, weighing 12-18 kg were used in the present study.

The experimental procedure followed was a modification of the original Folts' model (25). Baboons were anaesthetized with ketamine hydrochloride (10 mg/kg, i.m.), intubated with a cuffed endotracheal tube and ventilated by a respirator with oxygen supplemented with 0.5% Fluothane to maintain anaesthesia. Body temperature was maintained at 37°C with a heating table. A catheter was placed in a femoral vein for drug administration and blood sampling. A segment of another femoral artery was gently dissected free from surrounding tissue and a perivascular ultrasonic flow probe (Transonic Systems Inc., New York, NY) was placed around the distal dissection site. The mean and phasic blood flow were recorded continuously throughout the experiment. Baboons were allowed to stabilize for 30 min. Then the proximal dissection site of the femoral artery was injured by applying 3 occlusions of the artery for ten seconds with 2 mm interval using a spring-loaded forceps. A spring-loaded clamp next was placed in the middle of the injured site to produce an external stenosis of 65-80%. A gradual decline in blood flow due to platelet adhesion and aggregation was observed. When flow reached zero, blood flow was restored by pushing the spring of the clamp to mechanically dislodge the platelet-rich thrombus. This repetitive pattern of decreasing blood flow following mechanically restoration was referred to as cyclic flow reductions (CFRs). Additional endothelial injury and appropriate external stenosis selection was repeated. Finally, stable CFRs were obtained in these baboons.

After a 60-minute control period of reproducible CFRs (t=-60 min to 0 min), test agents (saline or mAb 82D6A3) were given via an intravenous bolus injection (t=0) and

monitoring was continued up to 60 minutes after drug administration (t=+60 min). The antithrombotic effect was quantified by comparing the number of CFRs per hour before and after drug administration. Blood samples for the different laboratory measurements (platelet count, coagulation, vWF occupation, vWF-collagen binding and plasma levels) were drawn at t = 0, +30, +60, +150, +300 min and 24, 48 hours after treatment.

Drug treatment: The doses of mAb 82D6A3 were selected on the base of preliminary dose-finding studies. In group I, two baboons were used as saline control. Three baboons, group II, received a dose of 0.1 mg/kg mAb 82D6A3, after 60 min recording, an additional 0.2 mg/kg mAb 82D6A3 was given. Since a preliminary study showed that mAb 82D6A3 has a long halflife, this therefore resulted in a final dose of 0.3 mg/kg. In group III, a dose of 0.6 mg/kg mAb 82D6A3 was given to two baboons. All agents were diluted with saline.

Platelet count, coagulation and bleeding time

All blood samples were collected into a plastic syringe containing a final concentration of 0.32% trisodium citrate. The platelet count was determined using a Technicon H_2 blood cell analyzer (Bayer Diagnostics, Tarrytown, NY).

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured at 37°C using a coagulometer (Clotex II, Hyland).

The template bleeding time was measured at the surface of the forearm using the Simplate® II device (Organon Teknika, Durham, NC). The volar surface of the forearm was shaved, and a pressure cuff was applied and inflated to 40 mmHg. Time elapsed until the visual cessation of blood onto the filter paper was recorded as the bleeding time. Bleeding times were followed for up to ten minutes.

Plasma concentration of 82D6A3

Microtiter plates (96-well, Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with 5µg/ml (in PBS, 100 µl/well) goat anti-mouse IgG whole molecule (Sigma, St. Louis, MO). Plates were blocked with 3 % milk powder (PBS, 250 µl/well) for 2 hours at room temperature (RT). Frozen plasma samples were thawed and incubated for 5 min at 37 °C before addition to the plate. Dilution series of the samples (1/2 in PBS) were made and incubated for 2 hours at RT. Goat anti-mouse IgG labelled with horse radish peroxidase (HRP) were added and were incubated for 1 hour at RT. Visualisation was obtained with ortho-phenylenediamine (OPD, Sigma) and the colouring reaction was stopped with 4 mol/l H_2SO_4. The absorbance was determined at 490 nm. After each incubation step, plates were washed with PBS, 0.1 % Tween-20, three times after coating and blocking steps and twelve times elsewhere. The plasma concentration of mAb 82D6A3 in each sample was calculated from a standard curve. This curve was obtained by adding known amounts of mAb 82D6A3 to baboon plasma (free of antibody) and plating ½ dilutions in PBS (starting from 6 µg/ml).

VWF-Ag levels

Determination of the vWF-Ag levels was performed essentially as described (26). Briefly, microtiter plates were coated with a polyclonal anti-vWF-Ig-solution (Dako, Glostrup, Denmark). Plates were blocked with 3 % milkpowder and samples were added to the wells at 1/40 to 1/2560 dilutions (samples were diluted in PBS, 0.3 % milkpowder). Bound vWF was detected with rabbit anti-human vWF HRP antibodies (Dako). Visualisation and wash steps were performed as described above. vWF-Ag levels were calculated from a standard

curve obtained by adding 1/40 to 1/2560 dilutions to the coated wells of a human plasma pool, known to contain 10 µg/ml human vWF.

vWF occupancy

Microtiter plates (96-well) were coated overnight at 4 °C with 125µl/well of a polyclonal anti-vWF-Ig-solution (Dako) (1/1000 in PBS). Plates were blocked with 3 % milk powder solution (in PBS, 250 µl/well) for 2 hours at room temperature (RT). Plasma samples were incubated for 5 min at 37 °C before addition to the plate. Pure samples were added and dilution series (1/2 in PBS) were made. Samples were incubated for 2 hours at RT. Samples containing 100 % occupied vWF were obtained by adding a saturating amount of mAb 82D6A3 (6 µg/ml) to the corresponding baboon plasma. Bound mAb 82D6A3 was detected by addition of goat anti-mouse IgG-HRP (1 hour at RT). Visualisation and wash steps were performed as described above. The vWF-occupancy of each sample was calculated as follows : (A490nm sample/ A490nm sample saturated with mAb 82D6A3)*100.

Determination of the vWF-collagen binding activity

The ELISA was performed essentially as described (26). Briefly, microtiter plates were coated with human collagen type I (Sigma). Plates were blocked with 3 % milk powder solution (in PBS, 250 µl/well). Pure sample and ½ dilution series were added. Bound vWF was detected with rabbit anti-human vWF-HRP antibodies. Binding of baboon vWF to collagen in the different blood samples was compared to the binding of vWF in the blood sample taken at time zero (pre sample) which was set as 100 %.

Determination of vWF binding to collagen and inhibition by F(ab) fragment of 82D6A3.

A 96-well plate was coated overnight with human collagen type I or III or calfskin collagen type I (25 µg/ml) and blocked. 2.5 µg/ml of recombinant vWF was used in the binding experiments.. For the competition experiments, purified human vWF (0.5 µg/ml fc) or plasma (1/50 fc) was preincubated with a dilution series of 82D6A3 or its F(ab) fragment during 30 min in a preblocked 96-well plate. Then the mixtures were added to the blocked collagen-coated plate. After 90 min incubation bound vWF was detected with a polyclonal anti-vWF-HRP conjugated antibody (Dako, Glostrup, Denmark) and visualization was performed with orthophenylenediamine (OPD, Sigma). The reaction was stopped with 4 mol/L H_2SO_4 and absorbance was determined at 490-630 nm. In between each incubation step the plates were washed 3-9 times with PBS (0.1% Tween 20).

Flow experiments.

Plastic thermanox coverslips were rinsed with 40 % ethanol and washed with water before spraying with human fibrillar collagen type I (100 µl (1 mg/ml) / coverslip). Blood was taken from healthy volunteers who had not taken aspirin or analogues for the last 10 days. The blood was anticoagulated with 25 U/ml low molecular weight heparin (LMWH) (Leo Pharmaceuticals, Vilvoorde, Belgium). The perfusion experiments were performed in a Sakariassen type flow chamber at 37°C, at wall shear rates of 600 s^{-1}, 1300 s^{-1} and 2600 s^{-1}. The perfusion chamber and tubings were rinsed with plasma during 20 min, and washed with 25 ml Hepes buffered saline (HBS) before starting the experiment. In each experiment 15 ml blood, preincubated for 15 min with an inhibitor as indicated, was perfused for 5 min. After the perfusion, coverslips were rinsed with 25 ml Hepes buffered saline and put in 0.5 % glutardialdehyde (10 min). Next the coverslips were placed in methanol (5 min), stained with May-Grünwald (3-5 min) and Giemsa (15-20 min) and washed 2 times with

destilled water. Coverslips were dried and analysed with an image analyser as described (29).

Isolation of MoAb binding phages.

Selection of phages was performed as follows. Biotinylated (see below) MoAb (10 µg) was bound to blocked streptavidin-coated magnetic beads (Dynal, Oslo, Norway). 2.10^{12} phages (PBS, 0.2 % milkpowder) were first incubated with blocked streptavidine-coated beads for 1 hour to eliminate the streptavidin-binders. Next the phages were added to the MoAb containing beads and after 90 min the input phages were removed and the beads were washed 10 times with PBS (0.1 % Tween-20) to remove the non-specific binders. The bound phages were eluted with 0.1 mol/L glycine, pH 2.2, and the eluate was immediately neutralized with 1 mol/L Tris, pH 8. After amplification of the phages, additional rounds of panning were performed. Phages were amplified by infection of *Escherichia coli* TG1 cells and partially purified from the supernatant by polyethylene glycol precipitation. Individual phage bearing *E. coli* were grown in a 96- well plate, and the supernatant was tested for the presence of 82D6A3- binding phages. Phage DNA was prepared and sequencing reactions were performed according to the T7-polymerase sequencing kit (Pharmacia) using the primer 5'-TGAATTTTCTGTATGAGG-3'.

Measurement of phage binding to 82D6A3. A 96-well plate was coated overnight with purified 82D6A3 (10 µg/mL). After 2 hours blocking with 2% milkpowder, a dilution series of the individual phage clones in PBS with 0.2 % milkpowder was added to the wells and phages were incubated at room temperature for 90 min. Bound phages were detected

after a 1-hour incubation with a polyclonal anti-M13-HRP conjugated antibody (Pharmacia) and visualization was performed with OPD.

Specificity of phage binding to 82D6A3.

A 96-well plate was coated overnight with purified 82D6A3 (10 µg/ml). After 2 hours blocking with 2 % milkpowder a dilution series of vWF or recombinant A3 domain was added. After a 30 min preincubation, a constant amount of phages was added to the vWF/A3 containing wells. 90 min later bound phages were detected as described above.

Competition between different phage clones for binding to 82D6A3 was analysed as above, except that 2.10^{10}/ml biotinylated phages of clone 1 were mixed with various concentrations of phages from clone 2, after which bound biotinylated phages were detected with streptavidin-HRP and OPD.

MoAb and phages were biotinylated using NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

Immunoblotting of phages

Purified phage clones (2.10^{10}) were electrophoresed on a 10 % SDS-PAGE gel under reducing and non-reducing conditions and electroblotted to a nitrocellulose membrane. After blocking the membrane with 4 % skimmed milk in PBS, the membrane was incubated with 82D6A3 (2 µg/ml) during 90 min, followed by a 1 hour incubation with GaM-HRP and developed using the ECL detection system from Amersham (Buckinghamshire, England). After each incubation step the membrane was washed with PBS containing 0.05 % Tween80.

RESULTS

Antithrombotic effect

The frequency of the CFRs was not changed by injection of saline (107 ± 7%). A dose of 100 µg/kg mAb 82D6A3 resulted in a significant reduction of the CFRs by 58.3 ± 4.8% (Fig.1). From a dose of 300 µg/kg onwards the CFRs were completely abolished, and could not be restored by increasing intimal damage or increasing stenosis (Fig. 2).

Platelet count, coagulation and bleeding time

The platelet count was not significantly affected by injection of the different doses of mAb 82D6A3 (Table I). No significant changes of PT or aPTT were observed in any of the animals (data not shown). The bleeding time was slightly prolonged after injection of 300 µg/kg and 600 µg/kg mAb 82D6A3, but returned to baseline levels 5 hours later (Table I).

Ex vivo mAb 82D6A3 plasma concentration, vWF-Ag levels, vWF-occupancy and vWF-collagen binding

Plasma samples, taken after several time points (see Material and Methods) in each study, were analyzed for mAb 82D6A3 plasma levels, vWF-Ag levels, vWF-occupancy and collagen binding activity ex vivo.

Thirty minutes after injection of the different doses of mAb 82D6A3, a small decrease in vWF-Ag levels were observed, whereas an increase in vWF-Ag levels above baseline was consistently measured after 24h (Table II & III).

Measurement of the mAb 82D6A3 plasma levels revealed no decrease in mAb 82D6A3 plasma levels in the first 3 hours of the experiment. Then 69 %, 23 %, 7.6 % mAb 82D6A3

was present after 300 min, 24h and 48h respectively when 300 µg/kg mAb 82D6A3 was administered (Table II).

Injection of 100 µg/kg mAb 82D6A3 resulted in an ex vivo inhibition of the vWF-collagen binding of 31 % (blood sample taken after 1 hour) (Table II). At doses of 300 µg/kg and 600 µg/kg no interaction between baboon vWF and collagen was observed in samples taken up to 5 hours after the administration of the mAb. Blood samples taken 24 hours after the injection of the drug revealed a recovery of the vWF-collagen interaction (Table II).

At 300 min after administration vWF-occupancy was 80 % for the 100 µg/kg dosis and near 100 % for the 300 µg/kg and 600 µg/kg doses. vWF remained occupied for a long time : even 48h after the injection of mAb 82D6A3, still 63 % of the vWF was occupied with mAb 82D6A3 (Table II).

Relation between the ex vivo vWF-occupancy and collagen binding, the vWF-occupancy and 82D6A3 plasma levels and between vWF-Ag and 82D6A3 plasma levels

vWF-occupancy inversely correlated with vWF-binding to collagen: to obtain inhibition of vWF-binding to collagen, a vWF occupancy of at least 70 % was required, with complete inhibition at 90-100 % occupancy (Fig. 3). These data were confirmed by *in vitro* experiments, where different concentrations of mAb 82D6A3 were added to baboon plasma (Fig. 4): occupancy levels of up to 60 % resulted in little inhibiton of the vWF binding to collagen, while inhibition was observed when 70 %-100 % of the vWF-binding sites for the antibody were occupied.

A good relation between 82D6A3 plasma levels and vWF-occupancy was also obtained with a maximum vWF-occupancy from about 1 µg/ml 82D6A3 onwards (Fig. 5).

Characterization of 82D6A3 and its F(ab)-fragment both under static and flow conditions.

82D6A3 is an anti-vWF antibody that binds with high affinity to vWF (Kd: 0.4 nM) (30) to the SpI proteolytic fragment and the recombinant vWF-A3 domain. Both the MoAb and its F(ab) fragment are able to inhibit plasma or purified vWF-binding to human collagen type I in a specific and dose-dependent manner with an IC_{50} of 20 ng/ml for the MoAb and 1 µg/ml for the F(ab) fragment (Fig. 6). The vWF binding to human collagen type III and calfskin collagen type I was inhibited in the same way. Next, 82D6A3 and its F(ab) fragment were tested under flow conditions at different shear rates (600, 1300 and 2600 s^{-1}). At a shear rate of 1300 s^{-1}, both the intact MoAb and F(ab) completely inhibited platelet deposition at 1-5 µg/ml and 10 µg/ml resp. (Fig. 7a) and the inhibitory effect increases with the shear applied (Fig. 7b).

Epitope mapping of 82D6A3 by means of phage display.

2 peptide phage display libraries, a linear pentadecamer and a cyclic hexamer, were used. After three rounds of biopanning with the pentadecamer library individual clones were grown and tested for their ability to bind to 82D6A3 (Fig. 8a). To determine whether the phages were binding to the antigen-binding pocket of the antibody, binding phage-clones were subjected to a competition ELISA to test whether vWF and the A3 domain were able to compete with the phages for binding to the 82D6A3 (Fig. 8b). From the different inhibitory clones that were thus identified, the sequence was determined, which resulted in the identification of 2 sequences: GDCFFGFLNSPWRVC (L15G8) and RSSYWVYSPWRFISR (L15C5). Both sequences shared the same 4 aa sequence SPWR. However the affinity of the L15G8 phage for binding to the MoAb was higher than that of the L15C5 phage.

After four rounds of biopanning with the cyclic hexamer library, individual clones were checked for binding to 82D6A3 (Fig. 9a) and for inhibition by vWF and the A3 domain (Fig. 9b). From the phage-clones that did compete, ssDNA was prepared and the sequence determined. 8 out of 13 clones displayed CMTSPWRC (C6H5), 4 out of 13 CRTSPWRC (C6G12) and 1 had the CYRSPWRC (C6A12) sequence. These sequences can be aligned with the L15 sequences that also contained the SPWR sequence. The L15G8 and C6H5 phage did compete with each other for binding to 82D6A3 (Fig. 10), which let us conclude that the epitope SPWR may be part of the epitope of 82D6A3. Furthermore by immunoblotting of the L15G8 and C6H5 phages it was demonstrated that the two cysteins present in both clones are forming a disulfide bridge, necessary for recognition by 82D6A3 (Fig. 11). Both the L15G8 sequence and the C6H5 sequence could be tentatively aligned in the vWF sequence more especially within the A3 domain.

DISCUSSION

Platelet adhesion to a damaged vessel wall is the first step in arterial thrombus formation. The tethering of platelets by vWF to the collagen exposed in the damaged vessel wall is especially important under high shear conditions. Anti-thrombotic compounds that interfere with the GPIb-vWF axis have been studied in animal models and were shown to be effective (19;21).

The present study evaluated for the first time the antithrombotic effects of inhibiting the vWF-collagen interaction *in vivo*. For this purpose, we used a monoclonal anti-human vWF antibody mAb 82D6A3 that by binding to the vWF A3-domain inhibits vWF binding to fibrillar collagens type I and III. mAb 82D6A3 furthermore crossreacts with baboon vWF and inhibits baboon vWF binding to collagen type I under static and flow conditions (Depraetere et al., submitted). A modified Folts' model was used to evaluate the antithrombotic efficacy of mAb 82D6A3 under high shear conditions (25) in baboons. This model allows to study the cyclic flow reductions (CFRs) due to platelet-dependent thrombi forming at the injured, stenotic site of the artery. This cyclic flow model has been described as representing some of the events occurring in patients with unstable angina and useful for studying the mechanisms of unstable angina. This model also allows a reproducible pattern of recurrent thrombosis to be established and is widely accepted as very effective and clinically relevant in testing potential antithrombotic agents (27;28).

Administration of 100 µg/kg, 300 µg/kg and 600 µg/kg mAb 82D6A3 resulted in 58 %, 100 % and 100 % inhibition of the CFRs respectively (Fig. 2) which corresponded well with the 31 %, 96 % and 96 % (measured in the 60 min plasma samples) *ex vivo* inhibition of the vWF-collagen interaction (Table II & III).

None of the administered doses, even the highest one, 600 μg/kg, tested, resulted in severe prolongation of the bleeding time or in thrombocytopenia (Table I) nor were the vWF-Ag levels impaired (Table II & III). These results together with the *ex vivo* inhibition of the vWF-collagen interaction show that the observed inhibitory effect results of a specific inhibition of the vWF-collagen interaction.

The absence of major bleeding problems correlates with ou finding that the effect of mAb 82D6A3 on platelet adhesion to human collagen type I was more pronounced at higher shear rates. This confirms that the vWF-collagen interaction is especially important at high shear stress, in other words in the arterial system, which could explain the observation of only a minor prolongation of the bleeding time.

The present invention shows that inhibition of thrombus formation under high shear stress *in vivo* can not only be obtained by inhibiting the vWF-GPIb interaction but also by interfering with the vWF-collagen interaction. Although also a number of anti-platelet GPIb compounds were succesfully used without effect on platelet counts, the risk of inducing thrombocytopenia in some occasions can never be ruled out, as seen with GPIIb-IIIa blockers. A vWF-blocker obviously may be safer in this respect. Both kinds of antithrombotics have the advantage of blocking the first step in thrombus formation which might in addition have some beneficial action in preventing restenosis after PTCA or stenting, in contrast with specific GPIIb-IIIa blockers which only interfere after the platelets have been activated. Activated platelets do not only secrete platelet activating substances but also vasoactive compounds such as platelet derived growth factor, known to induce smooth muscle cell migration and proliferation resulting in restenosis.

It was also revealed that F(ab)-fragments of 82D6A3, directed to the A3-domain of vWF, also bind to vWF with high affinity and are potent inhibitors of the vWF-collagen interaction under both static and flow conditions.

Selection of antibody binding phages from two different phage display libraries, a pentadecamer and cyclic hexamer library, resulted in phages that bind to 82D6A3 in a dose-dependent manner. Moreover, vWF and the recombinant A3- domain were able to inhibit phage binding to the MoAb indicating that the phages bind at or near to the antigen-binding site of 82D6A3. Sequence comparison of the phage displayed peptides revealed that a consensus SPWR sequence was present in all phages selected. From these results we can conclude that the SPWR sequence may be a part of the 82D6A3 epitope. The SPWR-sequence could be aligned to the VPWN sequence (aa 980-983) within the A3 domain, and in the three dimensional structure of the A3-domain located in the vicinity of previously identified amino acid residues important for vWF-collagen interaction. Finding consistently the same 4 aa consensus sequence on the one hand indicates that this sequence really might be important in the antibody recognition

In conclusion, the present invention demonstrates that vWF-collagen interaction plays an important role in acute platelet-dependent arterial thrombus formation: blockade of vWF-collagen interaction by mAb 82D6A3 or antigen recognising fragments thereof can induce complete abolition of thrombus formation in the injured and stenosed baboon femoral arteries. Accordingly, the mAb 82D6A3 can be used as a compound for the prevention of acute arterial thrombotic syndromes or to manufacture medicines to prevention of acute arterial thrombotic syndromes.

LEGEND TO TABLES

Table I: Platelet count and bleeding time measured after administration of different doses of mAb 82D6A3 in baboons.

Values are mean data ± s_D, / : not determined.

Table II : *Ex vivo* mAb 82D6A3 plasma concentration, vWF-Ag levels, vWF-occupancy and vWF-collagen binding activity measured after administration of 100 and 300 µg/kg mAb 82D6A3 to baboons.

Data are mean data ± s_D, of n = 9 i.e. at each time point, the plasma samples were measured 3 times in three different ELISA's and this for the 3 animal experiments.

Table III : *Ex vivo* mAb 82D6A3 plasma concentration, vWF-Ag levels, vWF-occupancy and vWF-collagen binding activity measured after administration of 600 µg/kg mAb 82D6A3.

Data are mean data ± s_D, of n = 6 i.e. at each time point, the plasma samples were 3 times measured in three different ELISA's and this for the 2 animal experiments.

LEGEND TO FIGURES

Fig. 1 : Inhibition of CFR by mAb 82D6A3.

Representative records of CFRs showing the effect of a bolus injection of 100 µg/kg and 300 µg/kg mAb 82D6A3.

Fig. 2 : Inhibition of CFRs by mAb 82D6A3.

Different dosis of mAb 82D6A3 were administrated to baboons and the CFRs were measured for 60 min. Data represent the mean ± s_D with n = 3 for 0.1 and 0.3 mg/kg mAb 82D6A3 and n = 2 for 0.6 mg/kg.

Fig. 3: Relation between the *ex vivo* vWF-binding to collagen and vWF-occupancy

All mean data measured at the different time points in the three different dose studies were used (Table II and III).

Fig. 4: Correlation between the in vitro measurements of the vWF-binding to collagen and vWF-occupancy

The experiment is a representative of 2 experiments,

Fig. 5: Relation between the *ex vivo* vWF-occupancy and mAb 82D6A3 plasma levels.

All mean data measured at the different time points in the three different dosis studies were used (Table II and III).

Fig. 6: Inhibition of vWF binding to human collagen type I

Inhibition of vWF (final concentration 0.5 µg/ml) binding to human collagen type I (□), type III (•) or to calf skin collagen (Δ) by 82D6A3 F(ab). Plates were coated with 25 µg/ml, 100 µl/well collagen. Bound vWF was detected.

Fig 7 Inhibition of platelet deposition onto a human collagen type I

Fig. 7b: Inhibition of platelet deposition onto a human collagen type I coated surface in flow at a shear rate 2600 s^{-1}. Filled bar: no antibody, open bar: 3 µg/ml 82D6A3, hatched bars: different concentrations of 82D6A3 F(ab)-fragments.

Fig. 7b: Shear-dependent inhibition of platelet deposition onto a human collagen type I coated surface by 82D6A3:. filled bars: no antibody, open bars: 5 µg/ml 82D6A3 F(ab)-fragments.

Fig. 8 Binding of phage clones

Fig. 8a: Binding of phage clones L15G8 (●) and L15C5 (■) to microtiterplates coated with 10 µg/ml 82D6A3.

Fig. 8b: Inhibition of the binding of phages L15G8 (●) and L15C5 (■) to microtiterplates coated with 10 µg/ml 82D6A3 by vWF. Final concentration L15G8: 2.10^9/ml, L15C5: 8.10^9/ml. Bound phages were detected.

Fig. 9 Binding of phage clones

Fig. 9a: Binding of phage clones C6H5 (●), C6G12 (■) and C6A12 (▲) to microtiterplates coated with 10 µg/ml 82D6A3.

Fig. 9b: Inhibition of the binding of phages C6H5 (●), C6G12 (■) and C6A12 (▲) to microtiterplates coated with 10 µg/ml MoAb 82D6A3 by vWF. Final concentration of phages: 5.10^{10}/ml. Bound phages were detected.

Fig. 10: Inhibition of the binding of biotinylated C6H5-phages to microtiter plates coated with 10 µg/ml 82D6A3 by L15G8 phages

Inhibition of the binding of biotinylated C6H5-phages to microtiter plates coated with 10 µg/ml 82D6A3 by L15G8 phages. C6H5-phages were used at a final concentration of 2.10^{10}/ml. Bound biotinylated C6H5-phages were detected with streptavidin-HRP.

Fig. 11: Alignment of the vWF sequence with the phage sequences

Alignment of the vWF sequence with the phage sequences (: similarity, | identity).

REFERENCE LIST

(1) Folts JD, Schafer AI, Loscalzo J, Willerson JT, Muller JE. A perspective on the potential problems with aspirin as an antithrombotic agent: a comparison of studies in an animal model with clinical trials. J Am Coll Cardiol 1999; 33(2):295-303.

(2) McGhie AI, McNatt J, Ezov N, Cui K, Mower LK, Hagay Y, Buja LM, Garfinkel LI, Gorecki M, Willerson JT. Abolition of cyclic flow variations in stenosed, endothelium-injured coronary arteries in nonhuman primates with a peptide fragment (VCL) derived from human plasma von Willebrand factor-glycoprotein Ib binding domain [see comments]. Circulation 1994; 90(6):2976-2981.

(3) Sixma JJ, Wester J. The hemostatic plug. Semin Hematol 1977; 14(3):265-299.

(4) Kehrel B. Platelet-collagen interactions. Semin Thromb Hemost 1995; 21(2):123-129.

(5) Phillips DR, Charo IF, Scarborough RM. GPIIb-IIIa: the responsive integrin. Cell 1991; 65(3):359-362.

(6) Tcheng JE. Platelet glycoprotein IIb/IIIa integrin blockade: recent clinical trials in interventional cardiology. Thromb Haemost 1997; 78(1):205-209.

(7) Hanson SR, Sakariassen KS. Blood flow and antithrombotic drug effects. Am Heart J 1998; 135(5 Pt 2 Su):S132-S145.

(8) Coller BS. GPIIb/IIIa antagonists: pathophysiologic and therapeutic insights from studies of c7E3 Fab. Thromb Haemost 1997; 78(1):730-735.

(9) Du X, Beutler L, Ruan C, Castaldi PA, Berndt MC. Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. Blood 1987; 69(5):1524-1527.

(10) Modderman PW, Admiraal LG, Sonnenberg A, dem Borne AE. Glycoproteins V and Ib-IX form a noncovalent complex in the platelet membrane. J Biol Chem 1992; 267(1):364-369.

(11) Vicente V, Kostel PJ, Ruggeri ZM. Isolation and functional characterization of the von Willebrand factor- binding domain located between residues His1-Arg293 of the alpha-chain of glycoprotein Ib. J Biol Chem 1988; 263(34):18473-18479.

(12) Berndt MC, Ward CM, Booth WJ, Castaldi PA, Mazurov AV, Andrews RK. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. Biochemistry 1992; 31(45):11144-11151.

(13) Pareti FI, Niiya K, McPherson JM, Ruggeri ZM. Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. J Biol Chem 1987; 262(28):13835-13841.

(14) Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, Ijsseldijk MJ, Vink T, de Groot PG, Sixma JJ. A3 domain is essential for interaction of von Willebrand factor with collagen type III. Thromb Haemost 1996; 75(6):950-958.

(15) Peng M, Lu W, Beviglia L, Niewiarowski S, Kirby EP. Echicetin: a snake venom protein that inhibits binding of von Willebrand factor and alboaggregins to platelet glycoprotein Ib. Blood 1993; 81(9):2321-2328.

(16) Chang MC, Lin HK, Peng HC, Huang TF. Antithrombotic effect of crotalin, a platelet membrane glycoprotein Ib antagonist from venom of Crotalus atrox. Blood 1998; 91(5):1582-1589.

(17) Miller JL, Thiam-Cisse M, Drouet LO. Reduction in thrombus formation by PG-1 F(ab')2, an anti-guinea pig platelet glycoprotein Ib monoclonal antibody. Arterioscler Thromb 1991; 11(5):1231-1236.

(18) Dascombe WH, Hong C, Garrett KO, White JG, Lyle VA, Miller JL, Johnson PC. Artificial microvascular graft thrombosis: the consequences of platelet membrane glycoprotein Ib inhibition and thrombin inhibition. Blood 1993; 82(1):126-134.

(19) Cauwenberghs N, Meiring M, Vauterin S, van W, V, Lamprecht S, Roodt JP, Novak L, Harsfalvi J, Deckmyn H, Kotze HF. Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. Arterioscler Thromb Vasc Biol 2000; 20(5):1347-1353.

(20) Cadroy Y, Hanson SR, Kelly AB, Marzec UM, Evatt BL, Kunicki TJ, Montgomery RR, Harker LA. Relative antithrombotic effects of monoclonal antibodies targeting different platelet glycoprotein-adhesive molecule interactions in nonhuman primates. Blood 1994; 83(11):3218-3224.

(21) Yamamoto H, Vreys I, Stassen JM, Yoshimoto R, Vermylen J, Hoylaerts MF. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998; 79(1):202-210.

(22) Golino P, Ragni M, Cirillo P, Pascucci I, Ezekowitz MD, Pawashe A, Scognamiglio A, Pace L, Guarino A, Chiariello M. Aurintricarboxylic acid reduces platelet deposition in stenosed and endothelially injured rabbit carotid arteries more effectively than other antiplatelet interventions. Thromb Haemost 1995; 74(3):974-979.

(23) Zahger D, Fishbein MC, Garfinkel LI, Shah PK, Forrester JS, Regnstrom J, Yano J, Cercek B. VCL, an antagonist of the platelet GP1b receptor, markedly inhibits platelet adhesion and intimal thickening after balloon injury in the rat. Circulation 1995; 92(5):1269-1273.

(24) Hoylaerts MF, Yamamoto H, Nuyts K, Vreys I, Deckmyn H, Vermylen J. von Willebrand factor binds to native collagen VI primarily via its A1 domain. Biochem J 1997; 324 (Pt 1):185-191.

(25) Folts J. An in vivo model of experimental arterial stenosis, intimal damage, and periodic thrombosis. Circulation 1991; 83(6 Suppl):IV3-14.

(26) Vanhoorelbeke K, Cauwenberghs N, Vauterin S, Schlammadinger A, Mazurier C, Deckmyn H. A reliable and reproducible ELISA method to measure ristocetin cofactor activity of von Willebrand factor. Thromb Haemost 2000; 83(1):107-113.

(27) Ikeda H, Koga Y, Kuwano K, Nakayama H, Ueno T, Yoshida N, Adachi K, Park IS, Toshima H. Cyclic flow variations in a conscious dog model of coronary artery stenosis and endothelial injury correlate with acute ischemic heart disease syndromes in humans. J Am Coll Cardiol 1993; 21(4):1008-1017.

(28) Willerson JT, Yao SK, McNatt J, Benedict CR, Anderson HV, Golino P, Murphree SS, Buja LM. Frequency and severity of cyclic flow alternations and platelet aggregation predict the severity of neointimal proliferation following experimental coronary stenosis and endothelial injury. Proc Natl Acad Sci U S A 1991; 88(23):10624-10628.

(29) Harsfalvi J, Stassen JM, Hoylaerts MF, Van Houtte E, Sawyer RT, Vermylen J, Deckmyn H. Calin from Hirudo medicinalis, an inhibitor of von Willebrand factor binding to collagen under static and flow conditions. Blood 1995;85:705-711.

(30) Hoylaerts MF, Yamamoto H, Nuyts K, Vreys I, Deckmyn H, Vermylen J. von Willebrand factor binds to native collagen VI primarily via its A1 domain. Biochem J 1997;324 :185-191.

Dose	100 µg/kg (n=3)		300 µg/kg (n=3)		600 µg/kg (n=2)	
	Platelet count	Bleeding time	Platelet count	Bleeding time	Platelet count	Bleeding time
min	(10^3/µl)	(min)	(10^3/µl)	(min)	(10^3/µl)	(min)
0	286 ± 54	2.7 ± 0.4	286 ± 54	2.7 ± 0.4	335	1.8
30	292 ± 65	2.7 ± 0.4	265 ± 41	4.6 ± 0.6	320	3.5
60	289 ± 79	3.5 ± 2.1	287 ± 53	7.3 ± 2.5	313	5.5
150	/	/	309 ± 83	6.4 ± 3.1	356	5
300	/	/	282 ± 7	3.15 ± 1.2	334	3
24h	/	/	312 ± 46	3.25 ± 0.3	347	/
48h	/	/	306 ± 79	3	/	/

Table 1:

	vWF-Ag levels (µg/ml)		MoAb 82D6A3 levels (µg/ml)		vWF occupancy (%)		collagen binding (%)	
min	100 µg/kg	300 µg/kg	100 µg/kg	300 µg/kg	100 µg/kg	300 µg/kg	100 µg/kg	300 µg/kg
0	10.2± 1.7	10.2 ± 1.7	0	0	2.3 ±1.3	2.3 ± 1.3	101 ± 7	101 ± 7
30	10.2± 2.5	8.8 ± 1.4	0.4 ± 0.07	2.9 ± 0.3	80 ±10.8	102 ± 10.4	64 ± 7	4 ± 1
60	8.9 ± 1.4	9.1 ± 2.4	0.4 ± 0.1	2.8 ± 0.3	80 ± 2.4	99 ± 10.6	69 ± 9	4 ± 1
150		9.7 ± 2.7		2.6 ± 0.1		101 ± 7.6		4 ± 1
300		8.8 ± 0.1		2.0 ± 0.5		94 ± 0.9		4 ± 1
24h		12.8 ± 1.3		0.7 ± 0.2		74 ± 31		91 ± 18
48h		13.2 ± 0.8		0.2 ± 0.01		63 ± 7.8		93 ± 0

Table II.

	VWF-Ag levels (μg/ml)	mAb 82D6A3 levels (μg/ml)	vWF occupancy (%)	collagen binding (%)
0 min	14 ± 1.7	0	6.9 ± 0.1	100 ± 0
30 min	11.5 ± 0.9	4.5 ± 0.5	96 ± 1	4 ± 0.2
60 min	10.8 ± 0.1	4.8 ± 0.7	96 ± 0.2	3.5± 0.2
150 min	11.9 ± 1.8	3.8 ± 0.5	97 ± 4	3.52 ± 0.2
300 min	10.5 ± 0	3.8 ± 0.6	97	4
24h	22.9 ± 0	1.4 ± 0.01	88	45

Table III

Claims

We claim

1) A ligand for use as a medicament, wherein said ligand specifically recognises domain A3 of von Willebrand factor or an epitope of said domain A3.
2) A ligand against von Willebrand factor (vWF), for use as a medicament, wherein said ligand inhibits interaction of von Willebrand factor with collagen.
3) The ligand of claim 1 for use as a medicament, wherein said ligand inhibits interaction of von Willebrand factor with collagen.
4) The ligand of claim 2 or 3 for use as a medicament, wherein said collagen are fibrillar collagen fibers.
5) The ligand of claim 2 or 3 for use as a medicament, wherein said collagen is thrombogenic collagen.
6) The ligand of any of the claims 2 to 5 for use as a medicament, wherein the thrombogenic collagen is type I and type III collagen.
7) The ligand of any of the claims 2 to 6 for use as a medicament, wherein said collagen is exposed in a damaged blood vessel wall
8) The ligand of any of the claims 1 to 7 for use as a medicament, wherein said ligand does not directly block the GPIb-vWF axis or the GPIIb-IIIa receptor.
9) Any of the claims 1 to 8, wherein the ligand is an antibody.
10) Any of the claims 1 to 9, wherein the ligand is an antibody against A3 domain of von Willebrand factor or a fragment thereof.
11) Any of the claims 1 to 10, wherein the ligand is a monoclonal antibody or a fragment Fab, Fab' or F(ab')2 thereof or a homologue of said fragment.
12) Any of the claims 1 to 11, wherein the ligand is a monoclonal antibody, a fragment Fab, Fab' or F(ab')2 thereof or a homologue of said fragment, that specifically binds to A3 domain of von Willebrand factor or a fragment thereof.
13) Any of the claims 11 to 12, wherein the monoclonal antibody is a humanised antibody having only the hypervariable regions of non-human animal origin
14) Any of the claims 11 to 13, wherein the monoclonal antibody is a humanised antibody having only the hypervariable regions of rodent origin.

15) The monoclonal antibody of claim 11 or 14, for use as a medicament said monoclonal antibody or an antigen binding fragment or recombinant binding protein thereof having a reactivity substantially identical to the monoclonal antibody obtained from a cell line, that has been deposited with the Belgian Collections of Micro-organisms, under accession number LMBP 5606CB.

16) The ligand according to any of the claims 1 to 15 for use as a medicament, wherein said ligand does not induce severe decline of circulating vWF-levels or a severe decline in platelet count when administered to a primate by bolus intravenous administration at a dose up to 600 µg/kg.

17) The ligand according to any of the claims 1 to 16 for use as a medicament, wherein said ligand does not result in severe prolongation of bleeding time or does not induce thrombocytopenia when administered to a primate by bolus intravenous administration at a dose up to 600 µg/kg.

18) The ligand according to any of the claims 1 to 17 for use as a medicament, wherein said ligand does occupy vWF and inhibits vWF-collagen binding when administered at a therapeutically effective dose up to 600 µg/kg to a primate by bolus intravenous administration.

19) The ligand according to any of the claims 1 to 18 for use as a medicament, wherein said ligand does not induce severe decline of circulating vWF-levels, severe decline in platelet count, severe prolongation of bleeding time or thrombocytopenia and that does not drastically affect clotting time (Prothrombin Time (PT) or activated Partial Thromboplastin Time (aPTT)) and said ligand does inhibit vWF-collagen binding and induces increased vWF-occupancy when administered to a primate by bolus intravenous administration at a therapeutically effective dose up to 600 µg/kg.

20) Any of the claims 9 to 19, wherein the ligand is in an immunoconjugate with a thrombolytic agent.

21) Claim 20, wherein the immunoconjugate contains a thrombolytic agent or a recombinant variant or fragment thereof, the thrombolytic agent being selected from the group consisting of staphylokinase, tissue plasminogen activator, streptokinase, single chain streptokinase, urokinase and acyl plasminogen streptokinase complex.

22) The ligand of any of the claims 1 to 19 for use in a medicine, which in an individual by interfering with the vWF-collagen interaction inhibits platelet tethering to a blood vessel surface under high shear stress or at high shear rates.

23) The ligand of any of the claims 1 to 19, for use in a medicine that by interfering with the vWF-collagen interaction inhibits the first steps of thrombus formation in an individual.

24) The ligand of any of the claims 1 to 19, for use in a medicine that by interfering with the vWF-collagen interaction blocks the first steps of thrombus formation before platelet activation and platelet secretion of vasoactive compounds that induce smooth muscle cell migration and proliferation resulting in restenosis.

25) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment.

26) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent the formation of a non-occlusive thrombus.

27) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent the formation of an occlusive thrombus.

28) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent arterial thrombus formation.

29) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent acute coronary occlusion.

30) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to maintain the patency of diseased arteries.

31) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent restenosis.

32) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent restenosis after PCTA or stenting.

33) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent thrombus formation in stenosed arteries.

34) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent hyperplasia after angioplasty, atherectomy or arterial stenting

35) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent unstable angina.

36) The ligand of any of the claims 1 to 19, for use in an antithrombotic treatment to prevent or treat the occlusive syndrome in a vascular system.

37) A pharmaceutical composition, comprising a ligand according to any of the claims 1 to 36 in admixture with a pharmaceutically acceptable carrier.

38) A pharmaceutical composition according to claim 37, further comprising a thrombolytic agent in a form either for simultaneous or sequential use.

39) Use of a ligand of any of the claims 1 to 25 for the manufacture of a medicament for use in the treatment of a thrombotic disorder in an individual in need thereof.

40) Use of a ligand of any of the claims 1 to 25 for the manufacture of a medicament for use in an antithrombotic treatment of any of the claims 27 to 36.

41) Use of a ligand of any of the claims 1 to 25 for the manufacture of a medicament that in an individual by interfering with the vWF-collagen interaction under high shear stress inhibits platelet tethering to a damaged blood vessel surface.

42) Use of a ligand of any of the claims 1 to 25 for the manufacture of a medicament that by interfering with the vWF-collagen interaction inhibits the first steps of thrombus formation in an individual.

43) Use of a ligand of any of the claims 1 to 25 for the manufacture of a medicament that by interfering with the vWF-collagen interaction blocks the first steps of thrombus formation before platelet activation or before activated platelet secretion of vasoactivating compounds that induce smooth muscle cell migration and cell proliferation resulting in restenosis.

44) An antithrombotic agent, which binds with the A3 domain of von Willebrand factor or an epitope thereof, resulting in inhibition of interaction of von Willebrand factor with collagen and which in an individual does not induce severe bleeding disorders at a minimal medicinal effective dose to exhibit antithrombotic action.

45) The antithrombotic agent of claim 44 which does not induce severe decline of circulating vWF-levels or a severe decline in platelet count at a minimal medicinal effective dose to exhibit antithrombotic action.

46) The antithrombotic agent of claim 44 which does not result in severe prolongation of bleeding time or does not induce thrombocytopenia at a minimal medicinal effective dose to exhibit antithrombotic action.

47) The antithrombotic agent of claim 44 which does inhibit vWF-collagen binding and increases vWF-occupancy at a minimal medicinal effective dose to exhibit antithrombotic action.

48) The antithrombotic agent of claim 44, which not induce severe decline of circulating vWF-Ag, severe decline in platelet count, severe prolongation of bleeding time or thrombocytopenia and that does not drastically affect clotting time (Prothrombin Time (PT) or activated Partial Thromboplastin Time (aPTT)) and that does inhibit vWF-collagen binding and induces increased vWF-occupancy at a minimal medicinal effective dose to exhibit antithrombotic action.

49) The antithrombotic agent of any of the claims 44 to 48, wherein said antithrombotic is an antibody, monoclonal antibody or a fragment Fab, Fab' or F(ab')2 thereof or a homologue of said fragment.

50) A method of antithrombotic therapy in an individual, comprising administering to the individual at risk of thrombosis, a therapeutically effective amount of the antithrombotic agent that inhibits the binding of von Willebrand factor to collagen of a damage blood vessel wall.

51) The method of antithrombotic therapy of claim 50, wherein the effective amount of antithrombotic agent inhibits platelet tethering to a damaged blood vessel surface.

52) A method for screening and selecting a medicinal effective and acceptable antithrombotic agent which inhibits von Willebrand collagen binding comprising:

a) characterising agents which inhibit von Willebrand collagen binding.

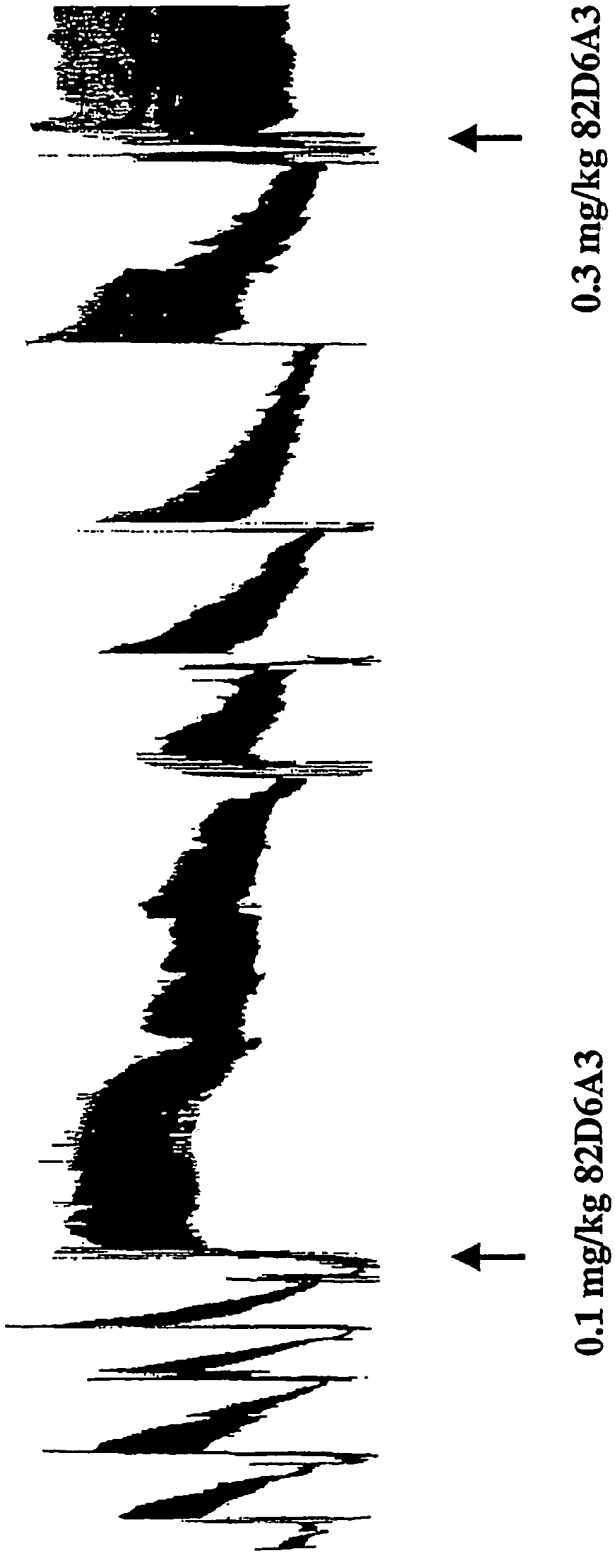
b) administering said agent to a mammal and preferably to a primate with injured blood vessel.

c) selecting the agents which at a dosis that significantly reduces cyclic flow reductions (CFR) do not drastically affect platelet count, do not drastically increase bleeding time, do not drastically change clotting time as measured by an assay such as activated Partial Thromboplastin Time or Prothrombin Time and do not drastically affect circulating vWF levels.

53) A polynculeotide encoding for the antigen binding Fab, Fab' or F(ab')2 fragment according to claim 12.

54) A DNA probe for detecting the polynucleotide sequence of claim 53, comprising a nucleic acid molecule having a sequence complementary to the coding sequence of said polynucleotide.

Figure 1



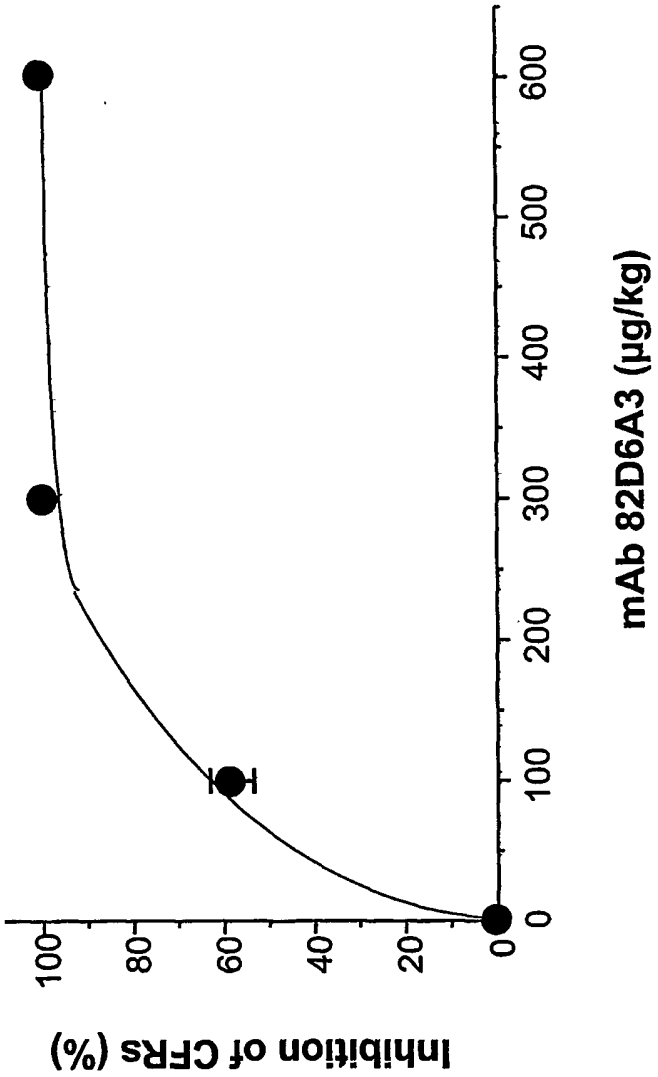

Inhibition of CFRs (%)
0
20
40
60
80
100
0
100
200
300
400
500
600
mAb 82D6A3 (µg/kg)

Fig2

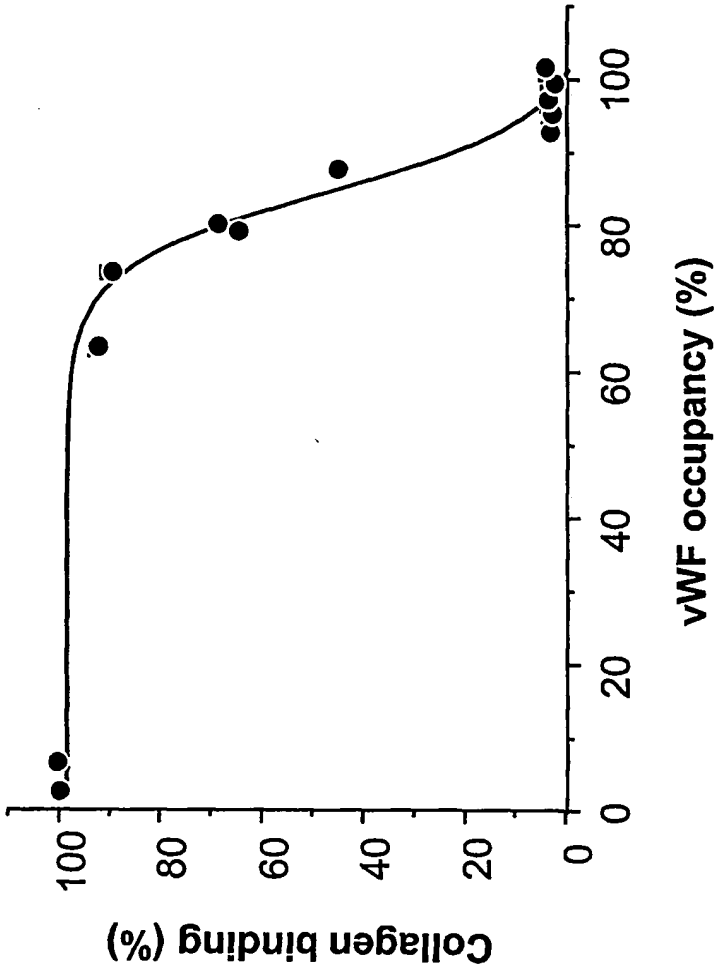

Collagen binding (%)
100
80
60
40
20
0
0
20
40
60
80
100
vWF occupancy (%)

Fig. 3

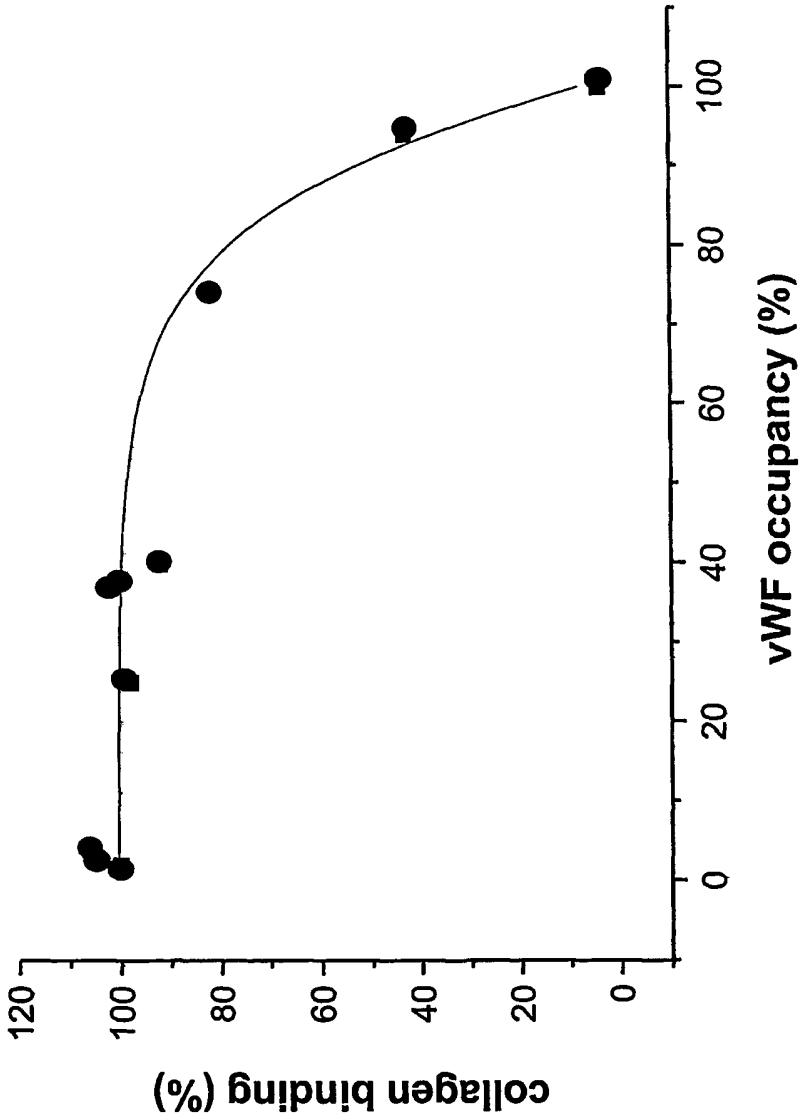

collagen binding (%)
120
100
80
60
40
20
0
vWF occupancy (%)
0
20
40
60
80
100

Fig4

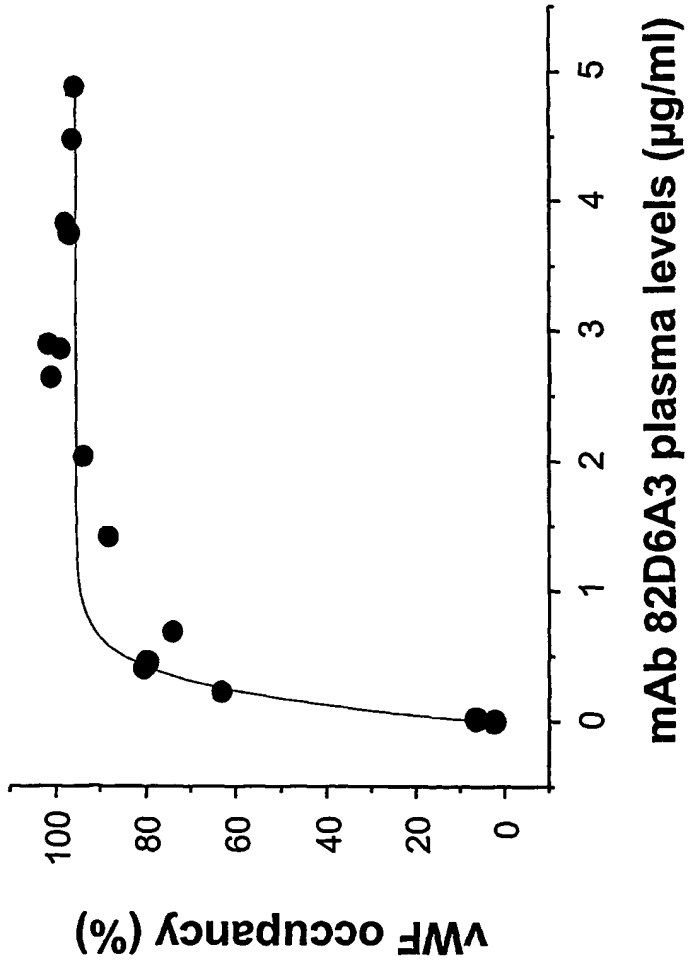

vWF occupancy (%)
0
20
40
60
80
100
mAb 82D6A3 plasma levels (µg/ml)
0
1
2
3
4
5

Fig. 5

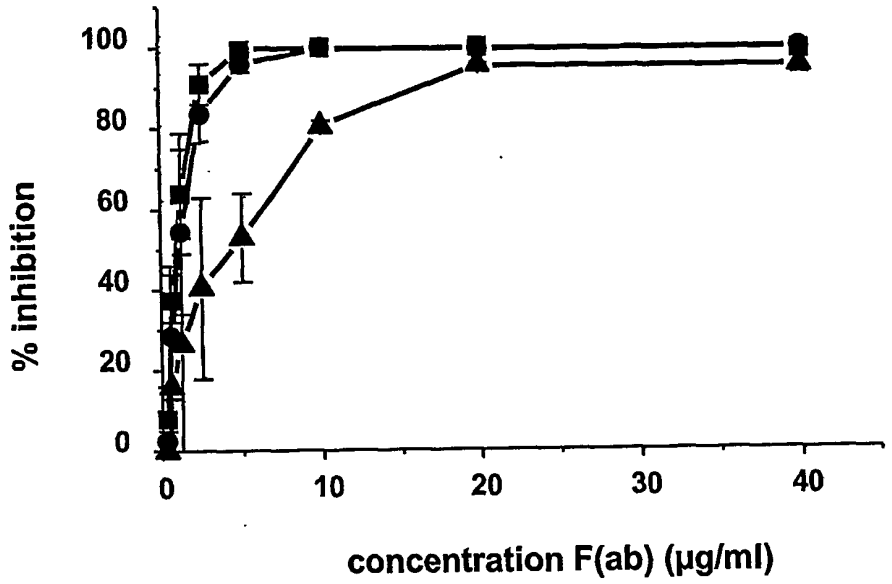
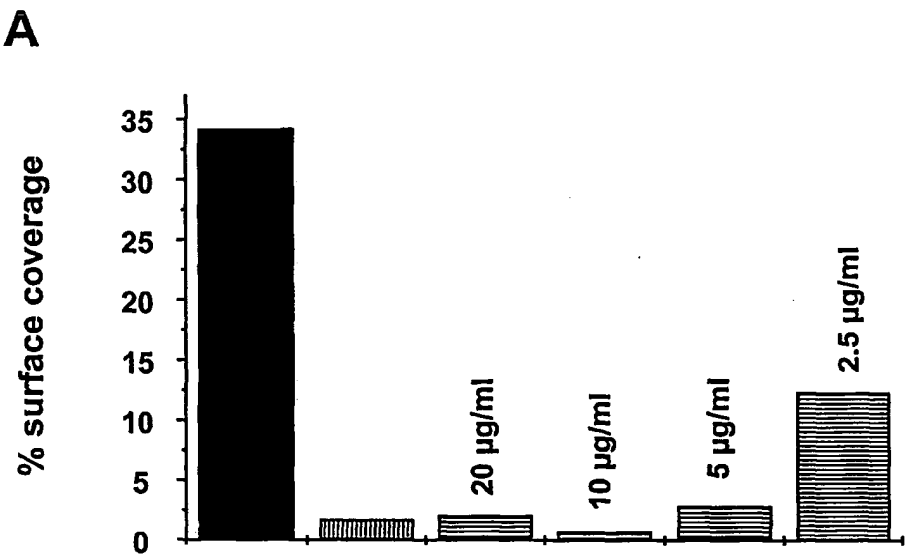

100
80
60
40
20
0
% inhibition
0
10
20
30
40
concentration F(ab) (µg/ml)

Fig. 6


A
% surface coverage
35
30
25
20
15
10
5
0
20 µg/ml
10 µg/ml
5 µg/ml
2.5 µg/ml

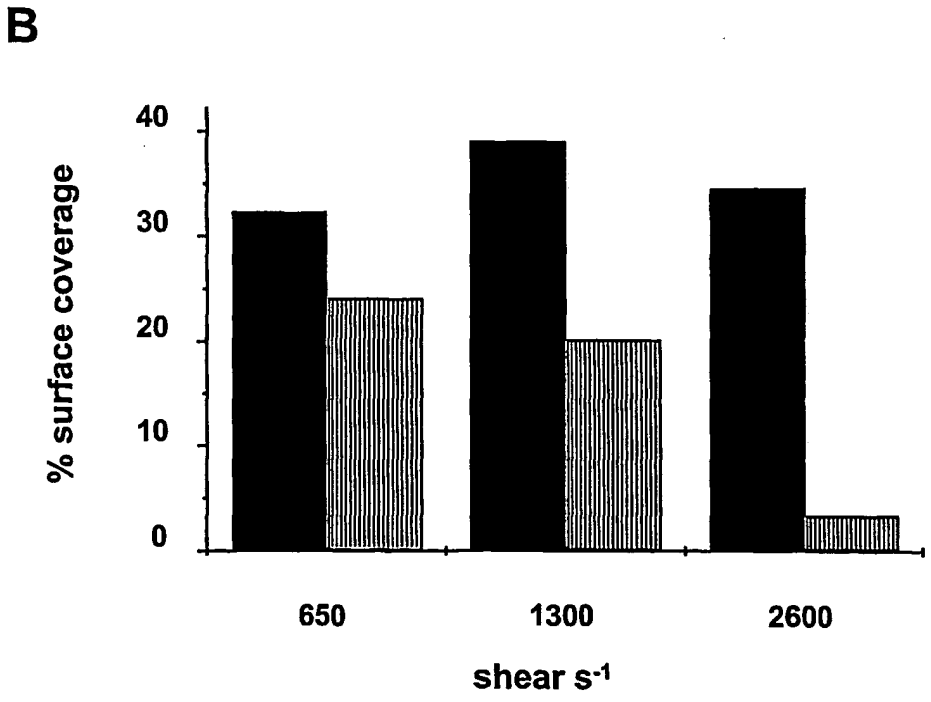
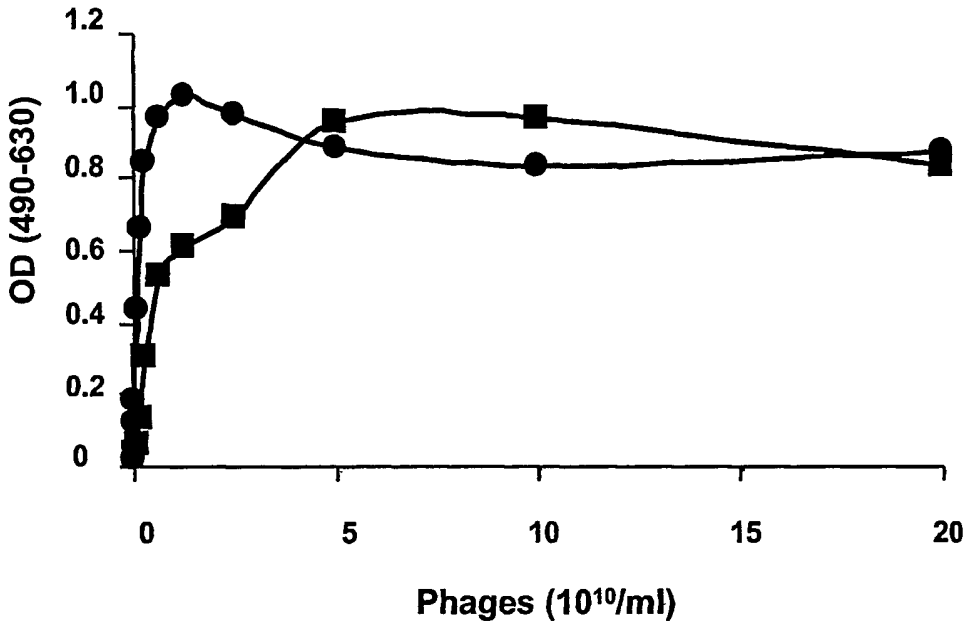

B
% surface coverage
40
30
20
10
0
650
1300
2600
shear s-1

Fig. 7a and 7b


1.2
1.0
0.8
0.6
0.4
0.2
0
OD (490-630)
0
5
10
15
20
Phages (10¹⁰/ml)

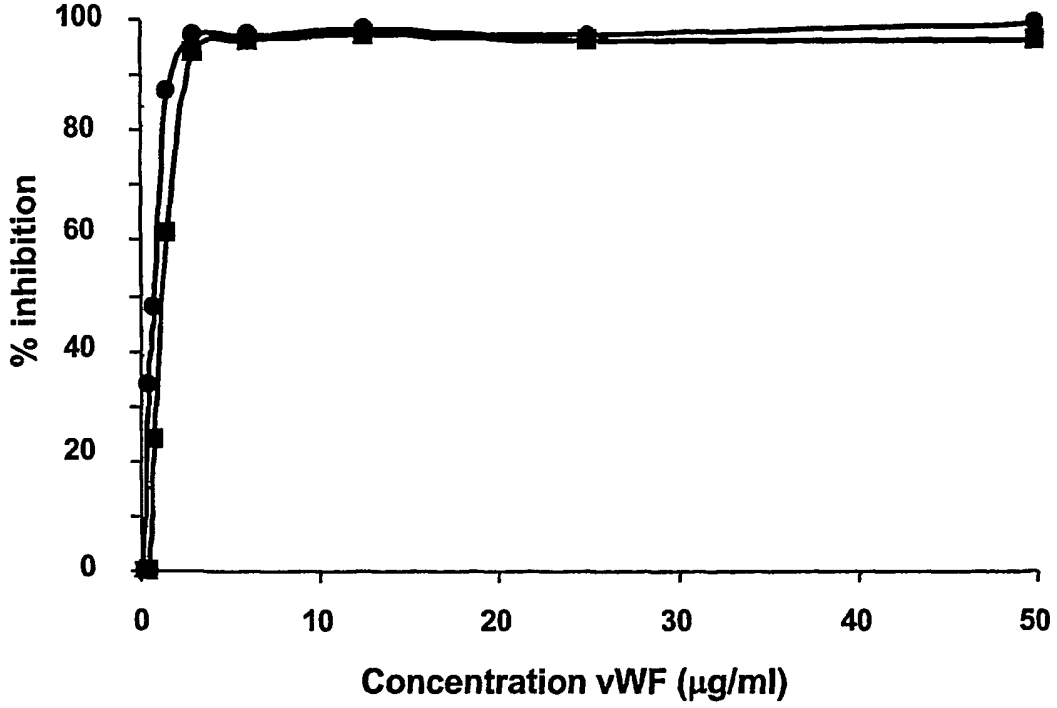
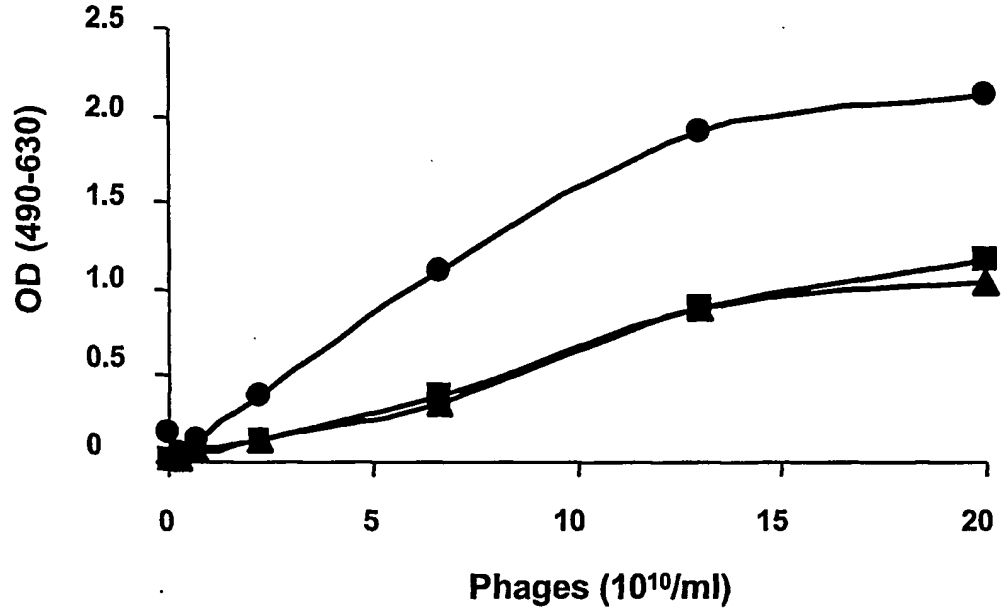

100
80
60
40
20
0
% inhibition
0
10
20
30
40
50
Concentration vWF (µg/ml)

Fig. 8a and 8b


2.5
2.0
1.5
1.0
0.5
0
OD (490-630)
0
5
10
15
20
Phages (10^{10}/ml)

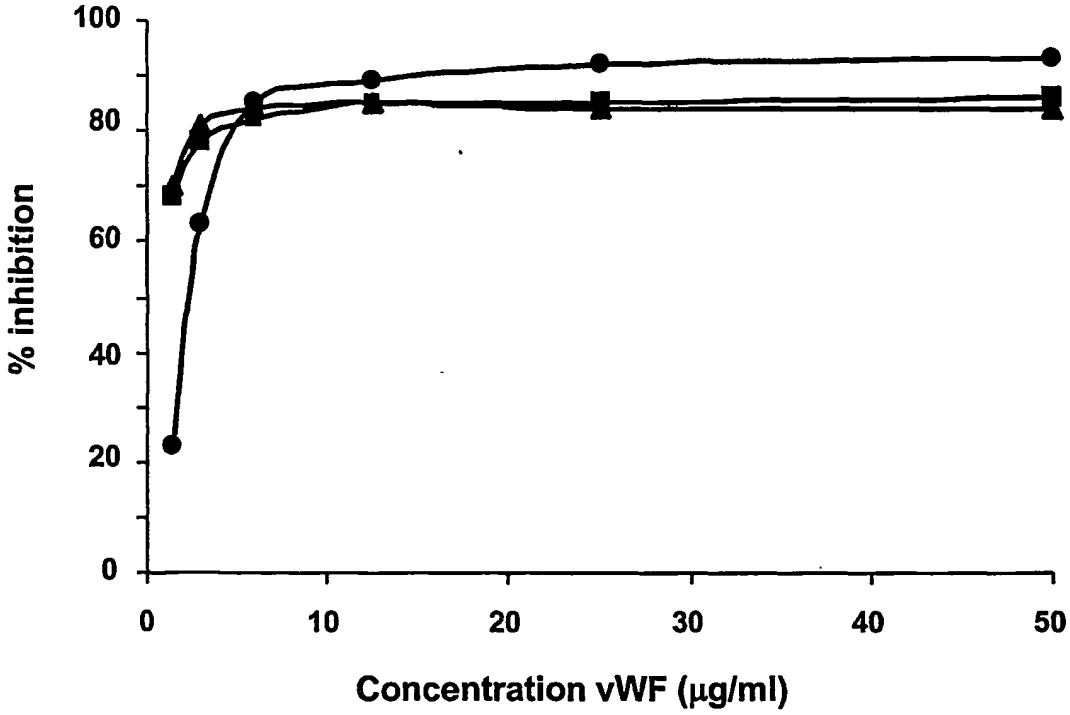

100
80
60
40
20
0
% inhibition
0
10
20
30
40
50
Concentration vWF (µg/ml)

Fig. 9a and 9b

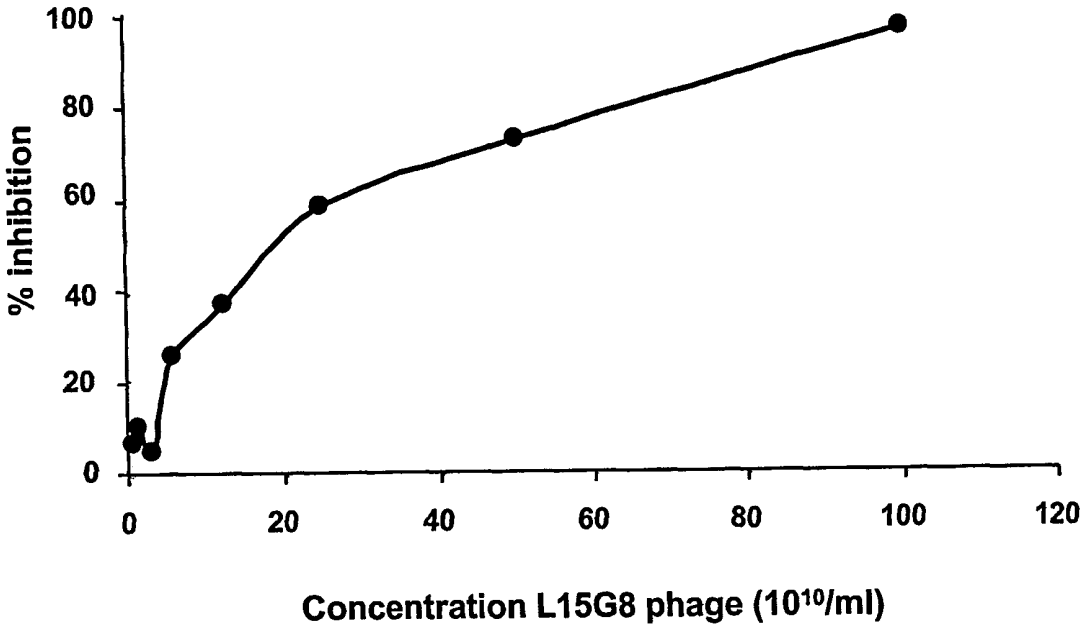

100
80
60
40
20
0
% inhibition
0
20
40
60
80
100
120
Concentration L15G8 phage (10^{10}/ml)

Fig. 10

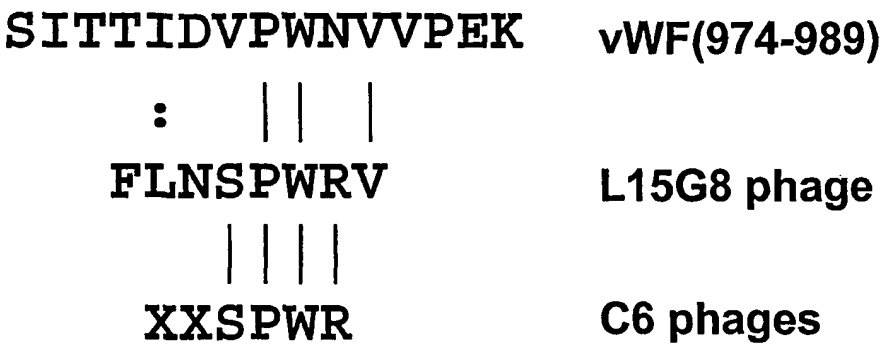

SITTIDVPWNVVPEK
vWF(974-989)
FLNSPWRV
L15G8 phage
XXSPWR
C6 phages

Fig. 11